

## Levels of Chemokine Receptors Expressed on Peripheral Blood T Lymphocytes of Egyptian Patients with Hepatocellular Carcinoma

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CC chemokine receptors (CCR) have an important role in the recruitment of leukocytes to the site of inflammation. The migration and metastasis of tumor cells shares many similarities with leukocyte trafficking, which is mainly regulated by chemokine receptor-ligand interactions. CCR1 and CCR5 are highly expressed in hepatocellular carcinoma (HCC) cells and tissues with unknown functions. In this study, we estimated the surface expression of chemokine receptors CCR1 and CCR5 on the lymphocytes of peripheral blood from patients with HCC in an attempt to identify their roles in tumorigenesis. The study was conducted on 52 patients of which, 24 of them with confirmed HCC and 28 with chronic hepatitis C virus infection. In addition, 20 apparently healthy controls with matched age and sex were also included in the study. All patient and control groups were subjected to the following: thorough history taking, clinical examination, abdominal ultrasonography and fine needle liver biopsy for patient's group when needed, complete blood count, liver function tests, viral markers for hepatitis B and C, serum alpha fetoprotein and flowcytometric detection of chemokine receptors CCR1 and CCR5 on peripheral blood T lymphocytes. The expression of chemokine receptors CCR1 and CCR5 on CD4+ and CD8+ T lymphocytes was significantly less in HCC and hepatitis C patient groups as compared to control group. Moreover, a significant decrease in the levels of CCR1 and CCR5 on CD8+ T lymphocytes was detected in HCC patients compared to patients with chronic HCV; however, it was not statistically significant for CD4+ cells. Furthermore in HCC patients, levels of CCR1 and CCR5 were significantly less in patients with large tumor size than small sized tumor. Data obtained showing reduced surface expression of CCR1 and CCR5 on CD4 and CD8 T lymphocytes reflect their possible role in altering the host's immune defense and disease pathogenesis, thus may be helpful for therapy design to ameliorate disease progression.

**H**CC is one of the most common cancers worldwide. Types of HCC vary by geographic location from a relatively rare tumor, like those found in North America and Europe, to a very common and highly malignant tumor that is characteristically encountered in Sub-saharan Africa and Southeast Asia (Srivatanakul *et al.*, 2004).

Hepatitis C virus is thought to be a non-cytopathic virus and liver damage is probably immune mediated. The vast majority of infected individuals develop persistent infection, suggesting that the immune system, while causing liver damage, is unable to mediate viral clearance. The mechanisms of viral persistence and disease pathogenesis are poorly understood but appear to be the result

of a complex interaction between the host immune system and the virus (Gane *et al.*, 2000).

CC-chemokines constitute a structurally related group of chemotactic cytokines which attract and activate specific subsets of inflammatory cells such as monocytes and T lymphocytes to the sites of infection (Laing & Secombes, 2004). CC-chemokines bind to specific G-protein coupled receptors to trigger cell activation and migration. In particular, the CC-chemokines CCL3 (macrophagic inflammatory protein 1 $\alpha$ , MIP-1 $\alpha$ ), CCL4 (macrophagic inflammatory protein 1 $\beta$ , MIP-1 $\beta$ ) and CCL5 (regulated upon activation, normal T cell expressed and secreted, RANTES) are ligands for the CC-chemokine receptor 5 (CCR5) and attract monocytes and

T lymphocytes (Azenshtein *et al.*, 2002; Ahlensteil *et al.*, 2004).

Chemokines play an important role in establishing the distribution of lymphocytes subpopulation in primary and secondary lymphoid tissues and in the recruitment of leucocytes into the sites of inflammation (Laing & Sercombe, 2004).

Lymphocyte traffic control is the main task of chemokines and chemokine receptors. Chemokine receptors not only regulate the chemotactic properties of lymphocyte that are crucial for the recruitment of lymphocytes from peripheral blood to the tumor, but also increase the effector functions of distinct lymphocyte subsets (Mehrad *et al.*, 2007).

The expression of chemokine receptors on tumor cells is associated with tumor progression (von Luetlichau *et al.*, 2008). The diverse biological roles for chemokines and their receptors in tumor growth and metastases have been identified (Vicari & Caux, 2002; Strier *et al.*, 2004; Laverdiere *et al.*, 2005). These actions include: modulation of tumor angiogenesis, tumor sensitivity to apoptosis, tumor proliferation, control of matrix degradation and the directed invasion of malignant cells during tumor metastasis (Lavergne *et al.*, 2004; von Luetlichau *et al.*, 2006).

*In vitro*, interleukin (IL)-8 and CXCL8 may be induced by the presence of X protein of hepatitis B virus and the hepatitis C virus NS5A (Liu *et al.*, 2003). Moreover, IL-8/CXCL8 protein was detected in hepatoma cells and in some malignant tissues. Although a decrease of chemokine receptors in HCC patients was reported, none of these studies did not clarify the exact role of those receptors in the tumor genesis (Liu *et al.*, 2004; Hirano *et al.*, 2007; Wu *et al.*, 2007 & 2008).

Still it remains elusive whether chemokine receptors are expressed in peripheral blood of HCC patients. We attempted to define the chemokine receptor expression namely CCR1

and CCR5 on the T lymphocytes of peripheral blood in patients with HCC as compared with levels of CC-receptors in chronic hepatitis C patients and healthy subjects to elucidate its possible role in HCC genesis.

## Patients and Methods

The present study included 52 patients selected from outpatient clinic and inpatient Hepatology Department of the National Liver Institute, Menoufiya University. The included patients were classified into 2 groups as following: group I included 24 patients with confirmed hepatocellular carcinoma diagnosed by clinical examination, abdominal ultrasonography, radiological examination and laboratory investigations. Liver biopsy was only done for some patients when needed. Their ages ranged from 49 to 68 years (20 males and 4 females). Group II included 28 patients with chronic HCV infection with no evidence for presence of HCC, diagnosed clinically, abdominal ultrasonography, laboratory investigations and liver biopsy, their age ranged from 25 to 60 years (19 males and 9 females).

Twenty healthy persons (16 males and 4 females) were also included in the study as a control group, age ranged from 38 to 57 years included 16 males and 4 females.

### Laboratory Investigations

Complete blood count (CBC) was performed using Sysmex automated cell counter. Liver function tests were done using a COBAS Integra-400 (Roche-Germany). Prothrombin time was done by Fibrinometer (Dade Behring-Germany). Alfa-fetoprotein protein was measured using an automated chemiluminescence system-ACS 180 (Chiron diagnostics Corporation, USA). Hepatitis viral markers were determined for anti-HCV antibodies by ELISA using Innogenetic N.V. (Ghent – Belgium) and HBV antigens by ELISA using Diasorin Kit (Diasorin SR, Italy). HCV RNA by RT-PCR using the automated Cobas Amplicor system of Roche (Amplicor PCR, Roche Molecular Systems, Germany).

Flowcytometric dual analysis of surface expression of CCR1 and CCR5 chemokines were assessed according to Ebert & Mc Coll (2002). CD4+ T lymphocytes and CD8+ T lymphocyte were measured by flowcytometry on coulter Epics XL (coulter electronics, Hialeah FL-USA) using FITC-conjugated monoclonal anti-human CD4 or anti-CD8 (Becton and Dickenson, USA). Negative isotopic method controls (Coulter Beckman, USA) were run to define non specific fluorescence. CCR1 and CCR5 were detected using PE-conjugated monoclonal anti-human CCR1

(Clone 53504) and PE-conjugated monoclonal antihuman CCR5 (Clone 45531) from Dako-Denmark.

The marker analysis was done on EDTA whole blood, 50  $\mu$ l of whole blood was incubated with 5  $\mu$ l of each monoclonal of interest (either of CD4, CD8, CCR1 or CCR5), for 20 minutes in dark. Erythrocytes were lysed by adding 3 ml ammonium chloride (0.83%) with potassium chloride (pH 7.2) for 5 minutes at 37°C. Lymphocytes were electronically gated using forward versus side-angle scatter. T lymphocyte subsets were identified by staining with anti- CD4 for T helper and with anti- CD8 for T suppressor. Two gates were drawn on CD4+ lymphocytes and the percentage of cells co-expressing CCR1 and CCR5 were recorded. Similarly, another two gates were drawn on CD8+ lymphocytes and the percentage of cells co-expressing CCR1 and CCR5 were recorded. Negative isotopic matched controls (Coulter Beckman, USA) were run to define non specific fluorescence. The values expressed in %, when 20% of the cells expressed the marker, the case was considered positive for that marker.

#### Statistical Analysis

Data were statistically analyzed using SPSS computer program version 12.0. Mean  $\pm$  SD were used to

describe data. Mann Whitney test was used to compare non parametric data between two patient groups and ANOVA test to compare parametric data between more than two patient groups. The ( $X^2$ ) test was used to compare the percentage means. The correlation studies were done employing the Pearson's correlation (r).  $P>0.05$  was considered statistically non-significant.

## Results

As regard to liver function tests (ALT, AST, alkaline phosphatase, GGT, total bilirubin, prothrombin time) and alpha fetoprotein were significantly increased in HCV and HCC patients compared to control group. While, serum albumin, platelet and total leucocytic counts were significantly decreased in both HCV, HCC patient groups as compared to control group (Table 1). Also, All HCV and HCC patients were positive for anti-HCV antibodies, RT-PCR RNA and negative for HBs Ag (data not mentioned in the table).

Table 1. Comparison of Liver Function Tests, AFP, Platelet and TLC of the Studied Groups.

	HCV group (n=28)	HCC group (n=24)	Control group (n=20)	P
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
ALT ( $\mu$ /L)	54.2 $\pm$ 22.8	47.2 $\pm$ 9.1	17.9 $\pm$ 2.5	<0.01
AST ( $\mu$ /L)	48.2 $\pm$ 18.2	58.2 $\pm$ 11.2	20.1 $\pm$ 4.2	<0.01
ALP ( $\mu$ /L)	66.5 $\pm$ 17.2	88.5 $\pm$ 30.4	27.2 $\pm$ 4.5	<0.01
GGT ( $\mu$ /L)	52.7 $\pm$ 16.3	71.6 $\pm$ 19.4	28.1 $\pm$ 6.1	<0.01
T. bilirubin(mg/dL)	2.5 $\pm$ 1.43	2.9 $\pm$ 1.50	0.76 $\pm$ 0.31	<0.01
Albumin (g/dL)	2.9 $\pm$ 0.5	2.6 $\pm$ 0.7	4.1 $\pm$ 0.4	<0.01
Proth. time (sec)	16.2 $\pm$ 2.6	18.4 $\pm$ 3.9	11.7 $\pm$ 1.2	<0.01
AFP (ng/ml)	15.4 $\pm$ 5.2	3632 $\pm$ 3121	6.2 $\pm$ 3.7	<0.01
Platelets ( $\times 10^3/\mu$ l)	142.3 $\pm$ 64.2	121.6 $\pm$ 32.6	271.5 $\pm$ 85.6	<0.01
TLC ( $\times 10^3/\mu$ l)	5.2 $\pm$ 1.65	6.2 $\pm$ 3.11	8.72 $\pm$ 2.89	<0.05

$P<0.05$  is significant

A significant decrease ( $P<0.01$ ) in the levels of CD4+ T lymphocytes and CD4+/CD8+ ratio was observed in patients with HCC as compared to controls, while levels of CD8+ were not significantly different in both

groups. On the other hand, a difference in the levels of CD4+ and CD8+ cells and (CD4+/CD8+) was not significant ( $P>0.05$ ) between HCC and HCV infected patients (Table 2).

Table 2. Comparison between HCC, HCV and Control Groups as Regard T helper (CD4+), T suppressor (CD8+) and their Ratio.

	CD4+ T cells Mean±SD	CD8+ T cells Mean±SD	CD4+/CD8+ ratio Mean±SD
HCC group (n=24)	22.41±8.11 <sup>a</sup>	26.07±5.28	0.97±0.23 <sup>a</sup>
HCV group (n=28)	35.24±6.91	21.53±4.18	1.39±0.15
Control group (n=20)	38.15±5.81	22.15±5.07	1.82±0.21

$P<0.05$  is significant.

<sup>a</sup> $P<0.01$  HCC versus Control group

The level of CCR1 and CCR5 expressions on CD4+ T cells were decreased significantly in HCC group as compared to controls ( $P<0.05$  and  $P<0.01$  respectively). Similarly, a significant decrease ( $P<0.01$  and  $P<0.001$ ) in the level of these receptors was noticed on CD8+ T cells obtained from HCC patients for CCR1 and CCR5, respectively (Table 3 & Fig. 1).

The CCR1 and CCR5 expressions on CD4+ T cells as well as CD8+ were significantly ( $P<0.05$ ) decreased in HCV

group compared to the control group (Table 3 & Fig. 2). When we compared the expression of chemokines observed on CD8+ cells obtained from both patients groups, the CCR1 and CCR5 levels were significantly lower ( $P<0.05$  and  $P<0.001$ , respectively) in HCC group as compared to HCV group. Although, the level of these chemokines on CD4+ cells were lower in HCC than in HCV patients, but this decrease did not reach statistical significance (Table 3).

Table 3. CC Chemokine Receptors Expression on CD4+ and CD8+ T lymphocytes form HCV, HCC Patients and Controls

	HCV group (n=28) Mean±SD	HCC group (n=24) Mean±SD	Control group (n=20) Mean±SD
CCR1 on CD4+	3.92±1.87 <sup>a</sup>	2.12±0.89 <sup>a</sup>	6.52±2.56
CCR5 on CD4+	4.54±2.09 <sup>a</sup>	3.27±1.20 <sup>b</sup>	7.16±1.98
CCR1 on CD8+	7.11±3.79 <sup>a,d</sup>	4.23±1.88 <sup>b</sup>	15.17±7.32
CCR5 on CD8+	5.78±2.54 <sup>a,e</sup>	1.02±0.23 <sup>c</sup>	9.14±3.25

$P<0.05$  is significant

<sup>a</sup> $P<0.05$ , HCV versus Controls & HCC versus Controls

<sup>b</sup> $P<0.01$  HCC versus Controls & <sup>c</sup> $P<0.001$  HCC versus Controls

<sup>d</sup> $P<0.05$  HCC versus HCV, <sup>e</sup> $P<0.001$  HCC versus HCV

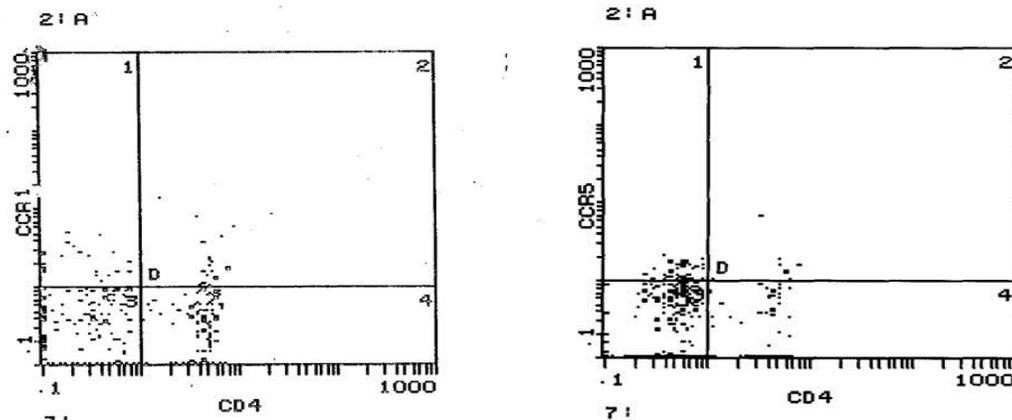


Figure 1. Expression of CCR1 on CD4+ & CCR5 on CD4+ in HCC patients

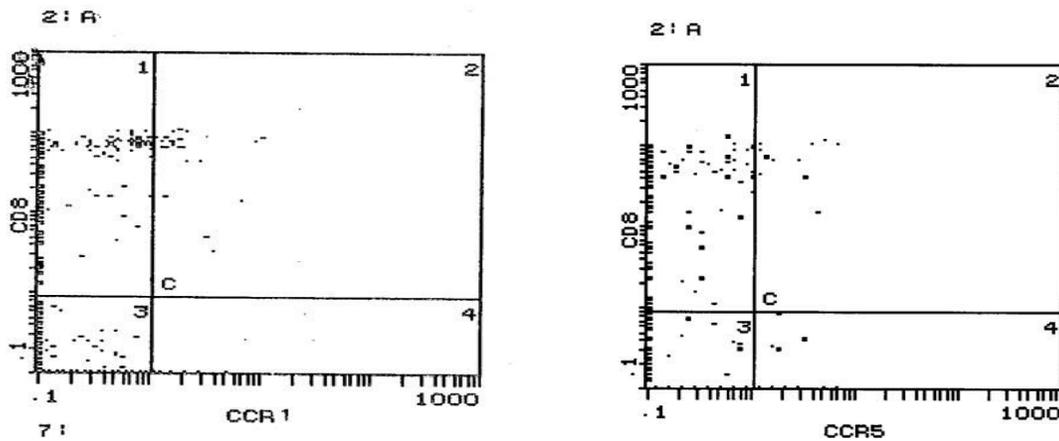


Figure 2. Expression of CCR1 on CD8+ & CCR5 on CD8+ in HCV patients

On the other hand, the levels of CCR1 and CCR5 expression on both CD4+ and CD8+ T cells were significantly decreased in HCC

patients with large sized focal lesion than those with small sized ones (Table 4).

Table 4. Comparison between Patients with Small and Large-Sized Focal Lesion as regard Chemokine Receptors.

	HCC group (n=24)		P
	Small sized tumor (n=14)	Large sized tumor (n=10)	
CCR1 on CD4+	2.29±0.56	1.03±0.64	<0.05
CCR5 on CD4+	4.07±1.58	2.11±0.76	<0.05
CCR1 on CD8+	5.03±1.67	2.18±0.93	<0.01
CCR5 on CD8+	2.16±0.84	0.97±0.52	<0.01

P<0.05 is significant.

## Discussion

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. It is a rapidly progressive malignancy with a dismal prognosis unless early diagnosis is done (Nguyen & Keeffe, 2004). Lymphocyte-mediated mechanisms play an important role in the host immune response against cancer (Goodman, 2007). Chemokines are considered pro-inflammatory mediators, potent immuno-modulators (lymphocyte activation and diversification), biological modifiers of erythrocyte function and angiogenic factors (Ko *et al.*, 2002; Allen *et al.*, 2007). To date, the relationship between pathogenesis of HCC and T lymphocyte immune mechanisms has not been established.

In an attempt to elucidate a possible role of chemokine receptors CCR1 and CCR5 in HCC genesis, we measured the levels of two chemokine receptors CCR1 and CCR5 expression on T cells obtained from patients with HCC as compared to patients with HCV and healthy controls.

The current study revealed a significant decrease in the levels of CD4+ T lymphocytes but not CD8+ in HCC in contrast to healthy controls. This result corroborates a previous study done by Liu *et al.*, (2004). It is expected in patients affected with liver cirrhosis that CD4+ and CD8+ cells migrate to the infected liver leading to decreased levels of such cells in peripheral blood. However, we did not observe in this study a significant decrease in the level of peripheral CD8+, this may be related to the degree of liver decompensation and the selection of our cases.

In this study, when comparing between HCV infected patients and HCC group, no significant difference between the two groups as regard the levels of CD4+, CD8+ cells and their ratio (CD4/CD8). This can be explained by that they are usually decreased in

peripheral blood of both groups by the same degree. This can be explained by our cases affected with HCC which may have developed from post viral hepatitis liver cirrhosis. We found in this study that CD4, CD8 and their ratio were lower in peripheral blood compared to the HCV group but did not reach statistically significance. This may be related to the selection of cases and the degree of hepatic decompensation, in which the ratio of CD4/CD8 is usually related to the progression of the liver disease. Moreover, Lechner *et al.*, (2000) recorded that in HCV patients the intracellular CD4+ and CD8+ T cells were increased leading to their decreased level in peripheral blood.

In our study, the CCR1 and CCR5 expressions on CD4+ T cells were significantly decreased in HCC group as compared to the control group. Moreover, decreased levels of these receptors were noticed on CD8+ T cells of HCC patients. This is in agreement with studies done by Shimizu *et al.*, (2001) and by Liu *et al.*, (2004) explaining that observed decreased levels of surface chemokine receptors expression may be due to receptor internalization into the cytoplasm after exposure to their ligands.

Hirano *et al.* (2007) reported that reduced expression of chemokine receptors CCR1 and CCR5 on circulating CD4+ and CD8+ was associated with liver compartmentalization of the chemokine receptor expression on CD4 and CD8. Such cells expressing these chemokine receptors may be recruited to liver tissues, a site of inflammation with concurrently decreased levels in the peripheral blood (Uekussa *et al.*, 2002).

Furthermore, when we compared HCC and HCV groups, the CCR5 expressions on CD4+ and CD8+ cells were significantly decreased in HCC patients. This is in agreement with the study done by Liu *et al.*, (2004) and also the study done by Uekusa *et al.*, (2002) who

proved that CCR5 expression on CD4 and CD8 were increased in liver tissue, concurrently with reduced expression in peripheral blood. This is explained by the fact that chemokine receptor CCR5 plays a critical role in T cell migration to cancer cells and subsequent induction of tumor regression and it is also involved in T cells migration to the inflamed liver and cirrhotic liver (Ebert & McColl, 2002; Lavergne *et al.*, 2004). In hepatoma tissues, CCR5 was detected on infiltrating lymphocytes but not hepatoma cells. Lu *et al.*, (2003) provided definitive evidence that hepatoma cells express CCR1 *in vitro* and *in vivo*. Because hepatoma cell lines express functional CCR1 in terms of adenylate-mediated cAMP changes, it is probable that CCR1-mediated signals may have some effects on hepatoma cells.

Promrat *et al.*, (2003) reported that in HCV infection, virus specific T cells must enter the liver from the circulation and then be retained at sites of infection in order to allow interaction with virus-containing hepatocytes. If the immune response is successful, infected cells will be cleared and the infiltrating lymphocytes will then die by apoptosis, leading to resolution. However, in chronic infection the antiviral response is ineffective but the inflammatory response persists, resulting in the retention and survival of lymphocytes in the liver, which mediate tissue damage and chronic hepatitis/fibrosis. In chronic inflammation, the balance is skewed in favor of accumulation with lymphocyte recruitment exceeding apoptosis. James & Timothy, (2006) added that chemokines are critical regulators of inflammation because they not only promote trans-endothelial migration of leucocytes, but also activate leukocyte integrins that are critical for their retention and survival at sites of infection or tissue damage.

In this study, we observed that the levels of CCR1 and CCR5 expression on both CD4+ and CD8+ T cells was significantly associated

with small sized focal lesions than the large sized in HCC patients. This is in agreement with the study done by Liu *et al.*, (2004) and Wu *et al.*, (2007) who found that the expression of chemokine receptors on peripheral blood lymphocyte in HCC patients exhibited a negative correlation with tumor size, indicating a relationship between the chemokine receptors expression on peripheral blood lymphocyte and tumor progression in HCC. This might be related to the sequestration of chemokine receptors positive T cells in large tumors or increased modulation of the immune response in HCC patients with large tumor burden. In the same manner, Wu *et al.*, (2008) recorded that, collectively, the CCL3-CCR5 axis appears to regulate intratumoral trafficking of leukocytes and fibroblasts, as well as MMP-9 and HGF expression, and as a consequence to accelerate neovascularization and subsequent metastasis formation.

In conclusion, CCR1 and CCR5 have come into prominence as a regulator of the inflammatory response through its influence over immune cell function. Moreover, CCR1 and CCR5 may play a role in tumorigenesis, therefore both chemokines would appear to represent a potential novel target for therapeutic intervention in the treatment of post-HCV liver disease or HCC. The significance of each receptor with respect to the course and outcome of the disease should be evaluated in a larger cohort of patients.

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